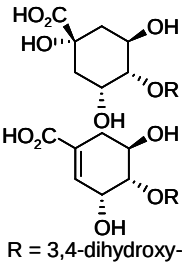
Graphical abstract



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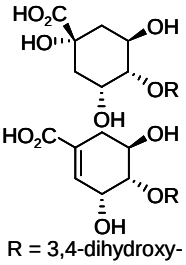

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Isolation, identification and quantitation of hydroxycinnamic acid conjugates, potential platform chemicals, in the leaves and stems of *Miscanthus* × *giganteus* using LC-ESI-MS^n

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Abstract

Miscanthus × *giganteus* is a source of platform chemicals and bioethanol through fermentation. Cinnamates in leaves and stems were analysed by LC-ESI-MS^n. Free phenols were extracted and separated chromatographically. More than twenty hydroxycinnamates were identified by UV and LC-ESI-MS^n. Comparative LC-MS studies on the leaf extract showed isomers of *O*-caffeoylquinic acid (3-CQA, 4-CQA and 5-CQA), *O*-feruloylquinic acid (3-FQA, 4-FQA and 5-FQA) and *p*-coumaroylquinic acid (3-*p*-CoQA and 5-*p*-CoQA). Excepting 3-*p*-CoQA, all were also detected in stem. 5-CQA dominated in leaf; a novel mandelonitrile-caffeoylquinic acid dominated in stem. Three minor leaf components were distinguished by fragmentation patterns in a targetted MS^2 experiment as dicaffeoylquinic acid isomers. Others (M_r 516) were tentatively identified as hexosylcaffeoyl-quinates. Three positional isomers of *O*-caffeoylshikimic acid were

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minor components. *p*-Hydroxybenzaldehyde was also a major component in stem. This is the first report of the hydroxycinnamic acid profile of leaves and stems of *M.* × *giganteus*.

Highlights

- *Miscanthus* × *giganteus* is an important source of biofuel and platform chemicals
- LC-ESI-MS^n was used to identify hydroxycinnamic acid conjugates in leaf and stem
- 24 hydroxycinnamates were identified in leaf, including caffeoylquinic acids
- 26 compounds were identified in stem, including mandelonitriles and mandelamides

Keywords: *Miscanthus* × *giganteus* leaf and stem tissue; hydroxycinnamic esters; UV; LC-ESI-MS^n; high-value chemicals.

1. Introduction

Society is demanding more green chemicals, which need to be sustainably sourced. The identification of high-value components in crops can help to increase value in the bio-refinery chain, similar to that in the oil industry. Current concerns about global warming and depletion of fossil fuels have stimulated interest in the development of cleaner technologies that use sustainable and carbon-neutral feedstocks (Alonso et al., 2010, Sims et al., 2006). *Miscanthus* is a perennial grass that is native in the tropical and subtropical regions of the world (Lewandowski et al., 2000). In the varying climatic regions of East Asia, different *Miscanthus* species have been identified (Lewandowski et al., 2000). The species *Miscanthus* × *giganteus* (*M.* × *giganteus*) is a large perennial hybrid resulting from a cross between *M. sinensis* and *M. sacchariflorus*. This rhizomatous grass with the C_4 synthetic pathway has been reported to have particularly high biomass potential with high output-to-input energy ratios (Clifton-Brown et al. 2008). Biomass is potentially a major source of renewable energy comprising of all organic material obtained from plants (including algae, trees and crops) (McKendry, 2002). Digestion, combustion or decomposition of the biomass releases chemical energy. In addition to providing energy, biomass feedstocks may also provide a source of commercially important chemicals and may reduce the reliance on petrochemical-derived products.

Plant phenols are secondary metabolites, which are ubiquitous in monocotyledonous and dicotyledenous plants (Parveen et al., 2010, Crozier et al., 2006, Ribéreau-Gayon, 2006, Lattanzio et al., 1994). These are synthesised *via* complex non-reversible biosynthetic pathways (*i.e.* phenylpropanoid and shikimate pathways) and often offer the plant protection against herbivory, microbial pathogen invasion, invertebrate pests and envir-

onmental stresses (Parveen et al., 2010, Crozier et al., 2006, Ribéreau-Gayon, 2006, Lattanzio et al., 1994, Nicholson, 1992). The family of hydroxycinnamic acids and their derivatives, known as the phenylpropanoids, is the most widely distributed group of naturally-occurring compounds (Parveen et al., 2010, Crozier et al., 2006, Ribéreau-Gayon, 2006). These compounds are characterised by the carbon skeleton C_6-C_3 (Parveen et al., 2010, Crozier et al., 2006). In higher plants, they are found in high concentrations and mainly consist of ferulic, caffeic, *p*-coumaric acids, while sinapic and 3.4-dimethoxycinnamic acid are encountered less frequently (Parveen et al., 2010, Crozier et al., 2006, Ribéreau-Gayon, 2006). In nature, the cinnamic acids usually occur in the *trans* geometrical isomer and most are in the form of esters, the most common being 5-*O*-caffeoylquinic acid (5-CQA) (Parveen et al., 2010, Crozier et al., 2006, Ribéreau-Gayon, 2006). Hydroxycinnamic acids can form esters with glycosides, glycerol, saturated long chain alcohols, fatty diols, fatty hydroxy acids and amino acids (Parveen et al., 2010). A large number of hydroxycinnamoyl conjugates have been reported in forage crops, grains, beverages and fruit and vegetable plants (Parveen et al., 2010, Crozier et al., 2006, Ribéreau-Gayon, 2006, Lattanzio et al., 1994).

There is much current interest in the potential of plant phenols (*e.g.* hydroxycinnamic acids, hydroxycinnamates and flavonoids) in the pharmaceutical industry (desirable properties include antioxidant, antimicrobial, anti-inflammatory, anti-cancer, anti-HIV, cholesterol-lowering activities and prevention of thrombosis and atherosclerosis) (Ou and Kwok, 2004, Galati, G. and O'Brien, 2004, Machado et al., 2008). Other attributes are useful in human nutrition (as chemopreventive agents) (Hodek et al., 2009), in the food and beverage industry (food additives, preservatives and flavouring agents) (Ou and Kwok, 2004), in the cosmetic industry (for photoprotection in skin lotions, sun-

screens, perfumes and hair creams) (Ou and Kwok, 2004), in the chemical industry (platform chemicals) (Corma et al., 2007) and in agriculture (in fruit sand vegetables) (Parveen et al., 2010, Crozier et al., 2006, Ribéreau-Gayon, 2006, Lattanzio et al., 1994).

Recently, high performance liquid chromatography-electrospray ionisation-mass spectrometry (LC-ESI-MS^n) has been used to characterise caffeoyl-, cinnamoyl- and feruloyl ester conjugates in many plant species (Clifford et al., 2003, 2005, 2006a,b,c,d 2007, 2008, Clifford and Knight, 2004, Parveen et al., 2008, Fang et al., 2002). *M.* × *giganteus* has been extensively investigated as a natural biofuel plant. However, little work has been carried out on the chemical composition of phenols in the leaves and stems of *M.* × *giganteus*. This knowledge would help determine the potential for exploiting this plant as a source of platform chemicals for industries and for thermal, biological and chemical conversion. Chemical composition is well known to affect the efficiency of conversion of biomass to energy and chemical products (Klinke et al., 2004). In particular, soluble phenols present in the biomass may be a limiting factor in the biotechnological conversion of sugars to ethanol (Klinke et al., 2004). In this study, we applied LC-ESI-MS^n to quantitative and qualitative profiling of hydroxycinnamic acid conjugates and flavonoids in leaf and stem extracts of *M.* × *giganteus*. Here we report the composition of hydroxycinnamates in the stems and leaves of this plant.

2. Results and discussion

2.1. Extraction of phenols

Free phenols were obtained from methanol extracts of fresh leaves and stems of *M.* × *giganteus*. An initial screening of the leaf extract by HPLC (Figure 1) identified *ca*. 20 phenolic constituents with characteristic UV shapes and UV absorptions (240-340 nm) where possible, typical of hydroxycinnamates (Figure 2). The mass spectra and retention times of the phenols were compared with those of standards including caffeic acid, *ortho*-coumaric acid, *para*-coumaric acid, cinnamic acid, ferulic acid and 5-caffeoylquinic acid. Lacking commercial standards, caffeoyl, *para*-coumaroyl and feruloyl conjugates were assigned by their parent ion, UV spectra, elution / retention time and their spectroscopic fragmentation data (MS^2 / MS^3). Quinate conjugates were identified by comparison of spectroscopic fragmentation patterns to those previously reported (Clifford et al., 2003, 2005, 2006a,b,c,d, 2007, 2008, Clifford and Knight, 2004, Parveen et al., 2008, Fang et al., 2002). HPLC-MS^n protocols have been developed to distinguish between positional isomers by fragmentation pattern (MS^2 and MS^3) and fragmentation mechanism (Clifford et al., 2003, 2005, 2008). In the absence of standards, all other identifications are considered provisional. Abundant hydroxycinnamates were quantified by absorption at 340 nm, following separation by reverse-phase HPLC. Caffeoylquinic esters were most abundant in leaf tissues with the 5-isomer comprising ≥90% of this fraction. Caffeoylquinic acid conjugated with cyano-compounds predominated in the hydroxycinnamate fraction in stem extracts (Table 1).

2.2. Characterisation of *O*-caffeoylquinic acids (M_r 354).

Esterification can occur at positions 1, 3, 4 or 5 of the quinic acid moiety, resulting in four positional isomers, 1-*O*-caffeoylquinic acid (1-CQA (**1**)), 3-*O*-caffeoylquinic acid (3-CQA (**2**)), 4-*O*-caffeoylquinic acid (4-CQA (**3**)) and 5-*O*-caffeoylquinic acid (5-CQA (**4**)). However, 1-acylated isomers are rare in higher plants (Parveen et al., 2010, Crozier et al., 2006, Ribéreau-Gayon, 2006, Lattanzio et al., 1994). 5-CQA was identified by comparison of retention time and MS with authentic material (Figure 2). Other positional isomers were assigned on the basis of the structure-diagnostic hierarchical keys developed by Clifford *et al*. (2005, 2006c,d, 2008), supported by UV spectra and retention times relative to 5-CQA . For minor components, data were derived from sensitive and selective fragment-targetted MS^n experiments (*i.e.* selected ion monitoring).

Three caffeoylquinic acids (CQAs) were detected in both the leaf and stem extracts and were assigned according to the fragmentation patterns of the mono-acylated isomers 3-CQA, 4-CQA and 5-CQA (Corma et al., 2007, Clifford et al., 2005, 2006a,b,c,d, 2007, Clifford and Knight, 2004). The diagnostic fragmentation patterns of 5-*O*-caffeoylquinic acid isomers in ESI-MS / MS in negative-ion mode involved cleavage of the caffeoyl and quinic acid moieties (Clifford et al., 2003). Two fragmentation pathways had previously been proposed in MS / MS (Clifford et al., 2003). Using ESI-MS^n in positive- and negative-ion modes, the three positional isomers were easily distinguished from one another due to differences in the relative intensities of ions in MS^2 and MS^3. In the leaf and stem extracts, *O-trans*-caffeoylquinic acids eluted in the order: 3-CQA, 5-CQA, 4-CQA under a 5-45% water (0.1% formic acid)-methanol gradient on a C_{18} reverse-phase column. 5-CQA was the most abundant caffeoylquinic acid, followed by

4-CQA, while 3-CQA was present as a minor component. The λ_{max} of 5-CQA was 324 nm, consistent with 3,4-dioxygenated cinnamate chromophores (Table 2). The predominant ions in the full MS negative-ionisation mode for 5-CQA were *m/z* 353 $[M - H]^-$ and 707 $[2 M - H]^-$ (Table 3). Fragmentation of the *m/z* 353 ion produced an MS^2 base peak *m/z* 191 $[quinate]^-$, followed by strong MS^3 ions at *m/z* 173 $[quinate - H_2O]^-$, 127 $[quinate – H_2O – HCO_2H]^-$, 111 [4-hydroxycyclohexa-1,3-dien-1-alkoxide]$^-$ and 93 $[phenoxide]^-$, respectively. A small *m/z* 179 $[caffeate]^-$ peak was identified in the MS^2 experiment, which, after fragmentation, yielded base fragment ion *m/z* 135 $[caffeate - CO_2H]^-$. These data are consistent with a dihydroxycinnamate (caffeoyl) unit (Parveen et al., 2008). Full MS in the positive-ionisation mode gave ions at *m/z* 377 $[M + Na]^+$, 355 $[M + H]^+$, 337 $[M - OH]^+$ and 163 $[M + H - quinic\ acid]^+$. MS^2 of the molecular ion *m/z* 355 ion gave *m/z* 163, which fragmented further to produce *m/z* 145 $[M + H - quinic\ acid - H_2O]^+$. Moreover, a typical fragmentation pathway resulted from ionisation at the carbonyl (Fang et al., 2002). A small ion was observed at 181 $[caffeic\ acid + H]^+$.

Stereoisomers were evident, probably geometrical *cis* / *trans* isomers of caffeic acid (Clifford et al., 2008). For example, a small peak at t_R 19.5 min (in both fractions) showed fragmentations identical to those of 5-CQA in MS / MS negative-ion mode spectra. This may be 1-CQA or the *cis*-5-CQA stereoisomer, as both are reported to fragment identically to *trans*-5-CQA (Clifford et al., 2008). Naturally occurring phenolic cinnamic acids occur in the *trans* configuration but exposure to UV light causes photochemical isomerisation to the *cis* configuration (Clifford et al., 2008). To test whether *cis* isomers were present, the leaf extract was split into two fractions; one control and one exposed to UV light (254 nm) for 30 min. ESI-MS / MS analysis of the UV-exposed extract showed increased relative intensity of the peak at t_R 19.5 min and

decreased intensity of the peak at t_R 12.7 min, in accordance with the observations of Clifford *et al*. (2008). The more hydrophobic *cis*-5-acyl isomer is reported (Clifford *et al*. 2008) to elute later than the *trans* isomer under a water / acetonitrile / acetic acid gradient; our data show a similar elution profile under a water / methanol / formic acid gradient. It was not possible to determine the UV absorption maximum for the *cis* isomer, owing to masking by co-eluting peaks. Geometrical stereoisomers did not influence the fragmentation of the positional isomers significantly, allowing identification of the *cis* / *trans* isomers (Clifford et al., 2008). In the stem fraction, a small but distinct peak with t_R 11.5 min showed properties very similar to those of 5-CQA and gave *m/z* 353 $[M - H]^-$. MS^2, MS^3 and MS^4 of the *m/z* 353 ion and its corresponding fragment ions showed very similar fragmentation patterns to those of 5-CQA. This was evidently a CQA-stereoisomer but 1-CQA was discounted, as this peak did not co-elute with an authentic sample of 1-CQA prepared by acid-catalysed hydrolysis of cynarin (1,3-dicaffeoylquinic acid) (Clifford et al., 2005).

Negative-ion mode ESI-MS of 3-CQA gave an abundant MS^2 peak with $[M - H]^-$ at *m/z* 353, yielding *m/z* 191 (100%) and abundant MS^3 ions at *m/z* 173, 127, 111, 93, 85. 3-CQA was distinguished from 5-CQA by an intense caffeate ion *m/z* 179 in MS^2 (Table 2). In contrast, 4-CQA was easy to distinguish due to its MS^2 base peak at *m/z* 173. Fragments *m/z* 191 and 179 were also present in relatively high intensities (see mechanistic discussion below). A small peak (t_R 4.4 min (leaf), (t_R 5.1 min (stem)) showed similar patterns of fragmentation to 3-CQA and was tentatively identified as *cis*-3-CQA. Likewise, a small peak at t_R 13.7 min (stem) was detected with similar fragmentation pattern to 4-CQA and was thus identified as the *cis* isomer.

2.3. Characterisation of *para*-coumaroylquinic acids (M_r 338).

Three monoacyl *para*-coumaroylquinic acids (3-*p*CoQA (**6**), 4-*p*CoQA (**7**) and 5-*p*CoQA (**8**)) were identified in leaf, while only the latter two were detected in the stem fraction. 5-*p*CoQA was the most abundant of the *p*-coumaroylquinic acids, followed by 4-*p*CoQA;3-*p*CoQA was comparatively minor in the leaf extract. These eluted in the order 3-*p*CoQA, 5-*p*CoQA, 4-*p*CoQA. Mass spectra of compounds **6**-**8** gave dominant ions at *m/z* 339 $[M + H]^+$ and 147 [M + H - quinic acid]$^+$. MS^2 of *m/z* 339 yielded an abundant ion at *m/z* 147 and a small ion at *m/z* 119 [M + H - quinic acid - CO]$^+$. Further fragmentation of *m/z* 147 yielded an MS^3 base ion at 119. In negative-ionisation mode, the three isomers gave the expected ions *m/z* 337 $[M - H]^-$ and 675 $[2 M - H]^-$. No UV spectra were recorded owing to masking by co-eluting peaks. MS^2 of *m/z* 337 at t_R 19.4 min (in leaf and stem) gave a base peak at *m/z* 191 [quinate]$^-$ and low intensity ion *m/z* 163 [coumarate]$^-$, characteristic of 5-*p*CoQA. We identify these as *trans*-5-*p*CoQA, as only *trans*-cinnamates occur as constituents of plants (Parveen et al., 2010, Crozier et al., 2006, Ribéreau-Gayon, 2006, Lattanzio et al., 1994). However, in ESI-MS, further signals were evident at t_R 26.7 (in the leaf extract) and t_R 26.6 min (in the stem extract) showing identical fragmentation patterns to 5-*p*CoQA; these are provisionally assigned as *cis*-5-*p*CoQA. Furthermore, in the stem fraction, a further peak with molecular ion *m/z* 337 $[M - H]^-$ was observed at t_R 17.7 min with patterns of fragmentation similar to the above compounds.

MS of 3-*p*CoQA gave an intense *m/z* 337 $[M - H]^-$ ion. MS^2 of this ion yielded a major ion *m/z* 163 [coumarate] and small fragment ions, *m/z* 191 [quinate]$^-$, and 119 [coumarate - CO_2]$^-$. No *cis* isomer was detected for 3-*p*CoQA. For 4-*p*CoQA, MS^2 of *m/z* 337

[M - H]$^-$ gave a base peak at *m/z* 173 [quinate - H_2O]$^-$ and a small ion at *m/z* 163 [coumarate]$^-$. The *cis* isomer eluted at t_R 15.2 min (UV absorbance maximum 324 nm), in contrast to the *trans* isomer which eluted at t_R 20.9 min. Targetted MS^2 experiments did not show dicoumaroylquinic acids or *para*-coumaroyl-amino acids in *M.* × *giganteus*.

2.4. Characterisation of *O*-feruloylquinic acids (M_r 368).

A targetted MS^2 experiments at *m/z* 367 in negative mode applied to both fractions identified three minor components 3-FQA (**10**), 4-FQA (**11**) and 5-FQA (**12**). The three positional isomers were identified by their distinct fragmentations; 3-FQA gave an intense MS^2 ion at *m/z* 193 [ferulate]$^-$, while 4-FQA yielded an abundant *m/z* 173 ion and weak ions *m/z* 193 and191. *Cis* isomers were also identified for both 3-FQA and 4-FQA (Table 2). In MS^2, the molecular ion *m/z* 367 (for 5-FQA) produced a strong base ion at *m/z* 191 [M - ferulic acid + H_2O - H]$^-$ and a weak ion at *m/z* 173. A small peak at t_R 31.3 min (leaf) and at t_R 31.2 (stem) was thought to be the *cis* stereoisomer. However, in the stem extract, a further peak of low abundance was detected at t_R 22.2 with similar spectroscopic data to 5-FQA; this may be 1-FQA (**9**). Full MS in positive-ion mode resulted predominantly in an ion of *m/z* 177 [ferulic acid + H - H_2O]$^+$ and smaller ions *m/z* 369 [M + H]$^+$ and 391 [M + Na]$^+$. Fragmentation of *m/z* 369 gave an intense *m/z* 177 ion and a smaller *m/z* 145 [ferulic acid - H_2O - OCH_3]$^-$ peak. Further fragmentation of *m/z* 177 yielded an intense base ion *m/z* 145. Selected ion monitoring experiments did not show di-feruloylquinic acids and feruloylamino acids in *M.* × *giganteus*.

2.5 Mechanistic Rationalisation of Fragmentation of O-Acylquinic acids.

O-Acylquinic acids **2-4**,**6-8**,**10-12** were distinguished on the basis of their retention times and recognition of fragmentation patterns from the *pseudo*molecular carboxylate anions. It was of interest to consider the chemical mechanistic basis of the different fragmentations. Clifford et al. (2005, 2006b) described fragmentations of the isomeric dicaffeoylquinic acids and other *O*-acylquinic acids, proposing mechanisms for acyl cleavages and β-elimination reactions. Scheme 1 shows the principal fragmentations of the three 3-acylquinates **2,6,10**, the three 4-acylquinates **3,7,11** and the three 5-acylquinates **4,8,12**. For each, two pathways are possible for loss of the acyl group – cleavage of the carbonyl–oxygen bond (which gives quinate *m/z* 191 and a neutral acyl equivalent) and β-elimination of a carboxylic acid (which gives a dehydrated quinate *m/z* 173 and coumarate *m/z* 163, caffeate *m/z* 179 or ferulate *m/z* 193 anions). Whereas Clifford proposed transfer of a proton as a critical step in each mechanism, analysis of the required reacting conformations suggests that this may not be the case. β-Elimination requires that the proton (removed by a base) and the leaving group be antiperiplanar for optimum overlap of orbitals. In a cyclohexane in a chair conformation, this can only occur when the proton and the leaving group (coumarate, caffeate or ferulate) are *trans*-diaxial. The predominant fragmentation of the 4-*O*-acylquinates **3,7,11** is β-elimination. In the chair conformation of **3,7,11** with the 4-acyloxy group axial (Scheme 1, central column), the proton at the 3-position is also axial and 1,3-*cis*-diaxial to the 1-carboxylate. This 1-carboxylate is perfectly located (6-membered cyclic transition state) to act as an intramolecular base to remove the 3-H and trigger the β-elimination, as shown. Indeed, an MM2-minimised model structure (Scheme 1, foot) shows that the base, the 3-H

and the leaving group are perfectly aligned. By contrast, there is very little β-elimination in the fragmentation of the 5-*O*-acylquinates **4,8,12** (Scheme 1, left column). In the required chair conformation (Scheme, lower left) with the *O*-acyl group axial, the neighbouring 4-H is *cis* (hence not antiperiplanar); the only proton antiperiplanar to the leaving group is 2-H, which is located on the opposite face of the cyclohexane to the base. Thus the 1-carboxylate cannot access this proton to trigger β-elimination and acyl cleavage (assisted by neighbouring group participation from 4-OH in the other chair conformation with 4-OH and 5-O-acyl *trans*-diequatorial) is the only option available. The 3-*O*-acylquinates **2,6,10** show mixed fragmentation (Scheme 1, right column). Antiperiplanar β-elimination triggered by the intramolecular base requires the boat conformation (Scheme 1, lower right), which is of higher energy than the chairs and thus less accessible. Thus the elimination fragmentations can be rationalised by participation of the 1-carboxylate as intramolecular base.

2.6. Characterisation of Dicaffeoylquinic Acids (M_r 516).

A targetted MS^2 experiment at *m/z* 515 in negative mode on the leaf extract detected three minor components which were distinguished by their patterns of fragmentation as 3,4-DiCQA (**13**), 3,5-DiCQA (**14**) and 4,5-DiCQA (**15**) (Clifford et al., 2003, 2005). These are very minor components and the UV absorbance maxima were masked by co-eluting peaks. Following separation by HPLC (C_{18} column) under conditions described above, these compounds eluted in the order 3,4-DiCQA, 3,5-DiCQA and 4,5-DiCQA between 42 and 52 min (Table 2 and 3). All three diCQAs gave the expected molecular ion *m/z* 515 $[diCQA - H]^-$ and MS^2 base peaks at *m/z* 353 $[caffeoylquinate]^-$. Consistent with previous studies, 3,4-diCQA and 4,5-diCQA gave MS^3 base peak at *m/z* 173

[quinate - H_2O]$^-$ and MS^4 base peak at *m/z* 93 [phenoxide]$^-$ (Clifford et al., 2003, 2005). Other secondary ions at *m/z* 299, 255, 203, 179 and 173 were detected; the data were consistent with previously published data (Clifford et al., 2003, 2005). Furthermore, 3,4-diCQA was distinguished from 4,5-diCQA due to the presence of the MS^2 fragment ion *m/z* 335 in 3,4-diCQA and the absence of this ion in 4,5-diCQA. 3,5-DiCQA was relatively easy to distinguish, owing to its MS^3 base peak *m/z* 191 and similar intensities of ions at *m/z* 179 and 191 with data previously published (Clifford et al., 2003, 2005). In ESI-MS^n, two possible fragmentation pathways have been proposed for the di-acylated compounds **13-15** (Clifford et al., 2003, 2005). Similarly, several compounds with M_r 515 [M - H]$^-$ were noted in stem but these could not be analysed further owing to limited sample.

Two minor compounds M_r 515 (M – H)$^-$ (**16**,**17**) were detected in leaf extracts which were clearly hydroxycinnamates but not dicaffeoylquinic acids. MS^2 experiments yielded major ions at *m/z* 455 [M – 2 HCOH - H]$^-$, 353 [5-*O*-caffeoylquinic acid - H]$^-$ and 323 [M - quinic acid - H]$^-$. Secondary ions were also observed at *m/z* 479 [M – 2 H_2O - H]$^-$ and 425 [M – 3 HCOH - H]$^-$. Further fragmentation of the *m/z* 353 ion yielded a base ion at *m/z* 191 and secondary ions at *m/z* 179 and 173. We tentatively identify these compounds as hexoside-caffeoyl quinic acids where the hexoside is linked to a hydroxy group on the caffeoyl moiety *via* an ester linkage; the linkage hexose–caffeoyl–quinic acid is demonstrated by the observation of fragment ions corresponding to CQAs.

2.7. Characterisation of caffeoyl-derivatives (M_r 336).

In the leaf extract, three small peaks at t_R 21.2 (**18**), 30.1 (**19**) and 23.8 min (**19**) yielded ions at *m/z* 335, corresponding to dehydrated caffeoylquinic acids (Table 2). All three

compounds yielded ions at *m/z* 179, 161 [caffeic acid – H - H_2O]$^-$ and *m/z* 135 characteristic of a caffeoyl moiety. The caffeic acid can be esterified at positions 3, 4, or 5 on shikimic acid to give three positional isomers. The MS^2 spectrum for compound **18** showed an intense ion at *m/z* 161, in contrast to the remaining two compounds. In addition, the MS^2 and MS^3 fragmentation data were consistent with synthetic 4-caffeoylshikimic acid (4-CSA) and compound **18** was assigned as 4-CSA (Jaiswal et al., 2010). The remaining two isomers were consistent with *cis-trans* isomers of 3-caffeoylshikimic acid (3-CSA) (**19**) (Jaiswal et al., 2010). Fragmentation pathways through radical anions have been claimed previously (Jaiswal et al., 2010), although the same fragmentations can be rationalised through diamagnetic anions (see below). In positive-ion mode, all three compounds gave an ion at *m/z* 175 [shikimic acid + H]$^+$.

Interestingly, two major peaks at t_R 25.7 and 27.5 min (M_r 485 (**20** and **21**)) and several minor peaks (M_r 503 (**24**-**27**) and M_r 485 (**22** and **23**)) were detected in the stem tissue which were absent in the leaf fraction (Figure 3). These compounds eluted at t_R 25.7, 27.5, 30.1 and 32.2 (M_r 485) and 15.5, 17.3, 18.9 and 19.4 (M_r 503). These compounds were detected in positive and negative ion modes at *m/z* 526 [503 + Na]$^+$, 486 [485 + H]$^+$, 502 [503 - H]$^-$ and 484 [485 - H]$^-$. While UV spectral characteristics were not typical of hydroxycinnamic acids, with maxima of 301 and 330 nm (M_r 485 compounds) and 302 and 332 nm (M_r 503 compounds), fragmentation patterns in MS^n experiments showed that both classes of compounds released 5-*O*-caffeoylquinic acid. These compounds are related since, in negative mode, *m/z* 502 eliminated H_2O to yield *m/z* 484. Further fragmentation of *m/z* 484 ion in MS^2 and MS^3 experiments yielded a major ion at *m/z* 310 [mandelonitrile–caffeate]$^-$. The 310 ion fragmented to yield a base ion at *m/z* 266 [310 - CO_2]$^-$, which further fragmented to yield an ion at *m/z* 239 [266 -

CN]$^-$. The 239 ion does not fragment further in negative mode, possibly indicative of an aromatic structure linked to the caffeoyl moiety. Based on fragmentation patterns from MS^n experiments and the observation that *p*-hydroxybenzaldehyde is abundant in stem, we propose that compounds with a molecular mass of 503 are conjugates (either diphenyl ethers or biphenyls) of *p*-hydroxyphenylacetamide or *p*-hydroxyphenylacetaldoxime radically coupled with the caffeic moiety of 5-CQA. We propose that the related M_r 485 $(M - H)^-$ compounds are derived from equivalent conjugates of *p*-hydroxymandelonitrile and 5-CQA. Halkier *et al.* carried out a study on the biosynthesis of the cyanogenic glucoside, dhurrin, by seedlings of *Sorghum bicolor* (Halkier et al. 1989). *p*-Hydroxymandelonitrile is glucosylated to dhurrin by a UDPG-glucosyltransferase and, in its absence, dissociates to *p*-hydroxybenzaldehyde and cyanide. These studies demonstrated that *p*-hydroxyphenylacetaldehyde oxime is an intermediate in the biosynthetic pathway to dhurrin. However no abundant ions corresponding to *p*-hydroxymandelonitrile (M_r 133), *p*-hydroxyphenylacetaldehyde oxime (M_r 151) or dhurrin (M_r 311) were observed in stem extracts. It cannot be ruled out that this compound was formed during extraction due to radical coupling of oxidised 5-CQA to cyano-compounds as a consequence of the high levels of polyphenol oxidase in *M.* × *giganteus* tissues.

3. Conclusions

LC-ESI-MS^n was applied to identify, characterise and quantify hydrocinnamates rapidly in the leaves and stems of *M.* × *giganteus*. More than twenty plant phenolics were identified. ESI-MS^n allowed detection of minute plant components, in addition to *cis / trans* geometrical isomers. Certain classes of hydroxycinnamic esters are relatively abundant in *M.* × *giganteus* tissues. This is relevant as this plant is a feedstock for platform chem-

icals and biological conversion to biofuels. Novel CQA-cyano compound conjugates in stem may have commercial value. To date, this is the first report on the hydroxycinnamate profile in the leaves and stems of *M.* × *giganteus*.

4. Experimental

4.1. Chemicals

Caffeic acid, *ortho*-coumaric acid, *para*-coumaric acid, cinnamic acid, ferulic acid and 5-*O*-caffeoylquinic acid standards were obtained from SigmaAldrich.

4.2. Plant materials

Miscanthus × *giganteus* was sown in experimental plots, in 2009, at the Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth, UK. Bulk plant material was harvested in July 2009, leaves and stems were separated, weighed and stored at -70°C for extraction, purification and analysis of phenols.

4.3. Extraction and purification of phenol substrates

Methanolic extracts were prepared as reported (Parveen et al., 2008). Free phenols were extracted from fresh *M.* × *giganteus* leaf material (*ca*. 400 g) in boiling water (500 mL) for 10 min. After cooling, the phenols were extracted with cold HPLC-grade methanol (Aldrich, UK); the extracts were combined and filtered and the solvent was evaporated to yield a brown residue (33.9 g). A subsample (460 mg) of this residue, in water (1.0 mL), was partly purified on Waters Sep-Pak (500 mg) C_{18} reverse-phase extraction cartridges, using a step-elution system with a water (with 5% acetic acid) / methanol series (100% water, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 100%

methanol). Following evaporation, the residues in each fraction, in 70% aqueous methanol, were centrifuged for 2 min at 10000 × *g*; the supernatant was decanted and the sample was subjected to HPLC analysis on a Thermo Finnigan HPLC/MS^n system for structural characterisation and quantification.

4.4. Preparative HPLC

A Waters HPLC system (Waters Corporation, USA) consisted of an auto-sampler, a pump, a photodiode array detector coupled with an analytical workstation. The column configuration consisted of a Waters C_{18} reversed-phase Nova-Pak C_{18} Radial-Pak column (4 μm, 8 mm × 100 mm). The sample injection volume was 10 μL. The detection wavelength was set at 240–400 nm, the flow rate was 1.0 mL min^{-1}, the auto-sampler tray temperature was kept at 4ºC and the column was at room temperature. The mobile phase consisted of water-acetic acid (A; 95:5, v/v) and HPLC grade methanol (B). The initial condition was A / B (100:0, v/v), the percentage of mobile-phase B increased linearly to 70% over the 35 min run. Phenols were detected with a PDA detector at two wavelengths (λ_{max} 280 and 340 nm) and individual peaks were collected and the solvent was removed by rotary evaporation. The software Empower (Waters Corporation, USA) was run on a Pentium III PC.

4.5. LC-ESI-MS^n analysis

The Thermo-Finnigan HPLC/MS^n system (Thermo Electron Corporation, USA) comprised an on-line degasser, an auto-sampler, a column oven, a pump and photodiode array detector coupled to an analytical workstation. The column configuration consisted of a

Waters C_{18} reversed-phase Nova-Pak column (4 μm, 3.9 mm × 100 mm). The sample injection volume was 10 μL.

The detection wavelength was set at 240-400 nm; the flow rate was 500 μL min^{-1}, with 50 μL min^{-1} going to the mass spectrometer. The auto-sampler tray temperature was kept at 4ºC and the column temperature was maintained at 20ºC. The mobile phase consisted of water-formic acid (A; 100:0.1, v/v) and HPLC grade methanol (B). The initial condition was A / B (95:5, v/v); the percentage of mobile-phase B increased linearly to 45% during 80 min. The software Xcaliber 1.4, 2003 (Thermo Electron Corporation, USA) was run on a Pentium IV PC and used to process the chromatographic data.

The instrumentation and chromatographic conditions of HPLC for LC-MS^n were the as described above. Following separation by HPLC, 400 μL min^{-1} was diverted to waste and 100 μL min^{-1} to the mass spectrometer. The mass spectra were acquired using a Finnigan LTQ linear ion trap instrument with an ESI source (Thermo Electron Corporation, USA). N_2 was used as the sheath and auxiliary gas and He was used as the collision gas. Highly sensitive targetted MS^n experiments were used to seek ions of low abundance, such as 1-CQA, dihydroxycinnamoylquinic acids and hydroxycinnamoylamino-acid conjugates. For the phenolic acids, the ionisation mode is negative and the interface and MSD parameters were as follows: sheath gas, 30 arbitrary units; auxiliary gas, 10 units; spray voltage, 4 KV; capillary temperature 320ºC; capillary voltage, -31 V; tube lens offset, -63 V. The ionisation mode in positive for the phenolic acids and the interface and MSD parameters were as follows: sheath gas, 30 arbitrary units; auxiliary gas, 5 units; spray voltage, 4.0 KV; capillary temperature 320ºC; capillary voltage, 23 V; tube lens offset, 50 V.

4.6. UV irradiation for *trans* → *cis* isomerisation

Aqueous methanol extracts (1.0 mL) of leaf and stem tissue of *M.* × *giganteus* were placed in a lightbox under a shortwave UV lamp (Sigma-Aldrich, UK) and irradiated at 254 nm for 30 min.

5. Acknowledgements

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Figure 1.

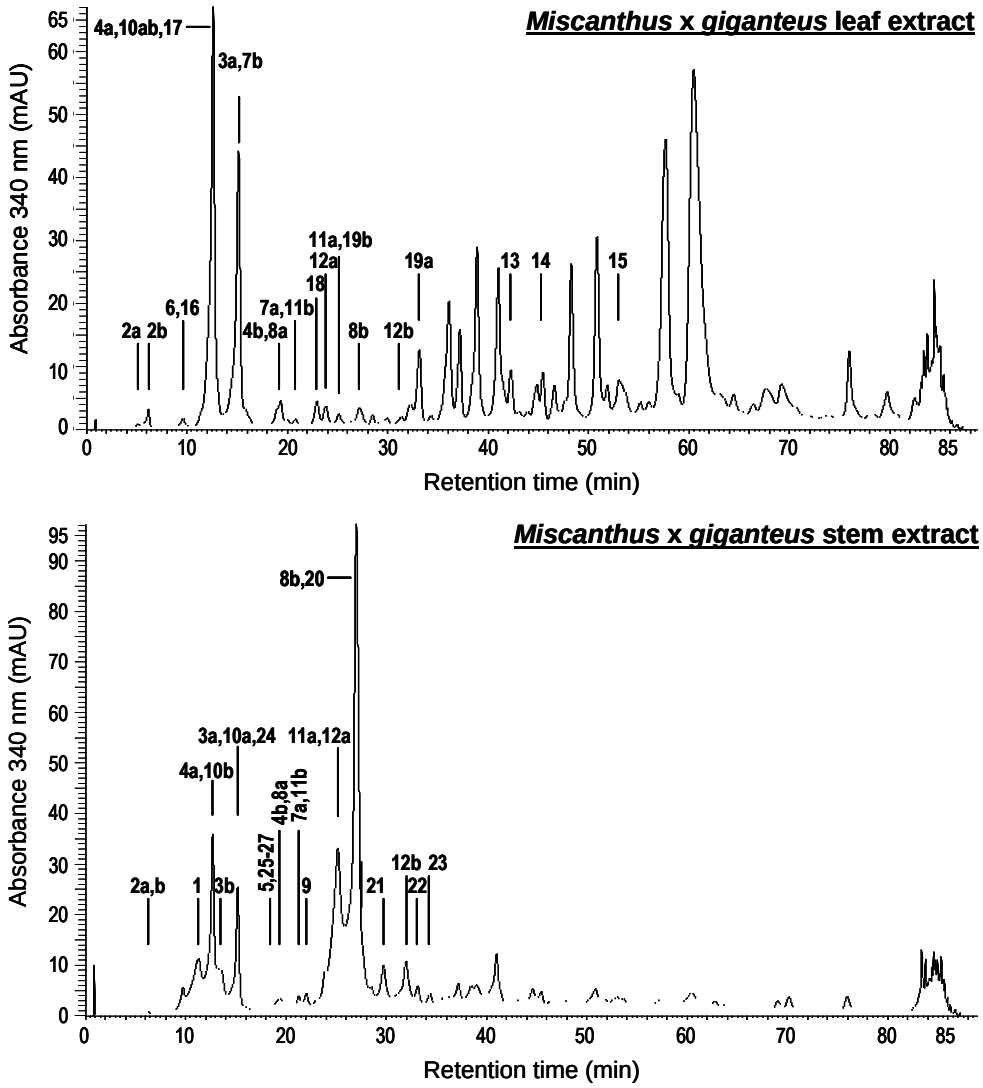
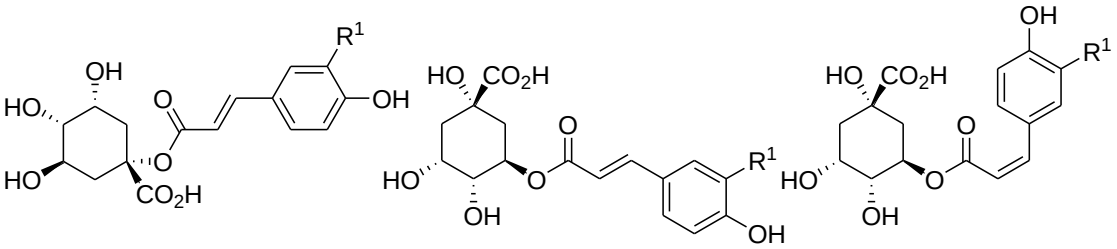

Miscanthus x giganteus leaf extract
Absorbance 340 nm (mAU)
Retention time (min)
4a,10ab,17
3a,7b
11a,19b
12a
18
19a
13
14
15
6,16
7a,11b
2a
2b
4b,8a
8b
12b
Miscanthus x giganteus stem extract
Absorbance 340 nm (mAU)
Retention time (min)
8b,20
3a,10a,24
11a,12a
4a,10b
4b,8a
7a,11b
5,25-27
12b
23
2a,b
1
3b
9
21
22

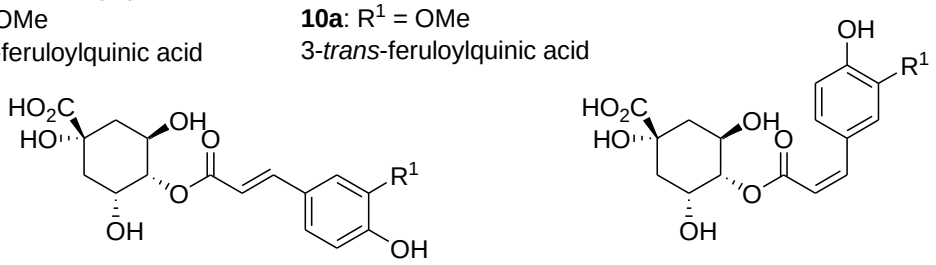
Figure 2.



1: R^1 = OH
1-*trans*-caffeoylquinic acid
5: R^1 = H
1-*trans*-coumaroylquinic acid
9:R^1 = OMe
1-*trans*-feruloylquinic acid

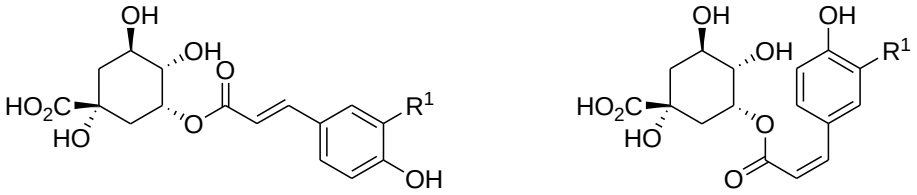
2a: R^1 = OH
3-*trans*-caffeoylquinic acid
6: R^1 = H
3-*trans*-coumaroylquinic acid
10a: R^1 = OMe
3-*trans*-feruloylquinic acid

2b: R^1 = OH
3-*cis*-caffeoylquinic acid
10b: R^1 = OMe
3-*cis*-feruloylquinic acid



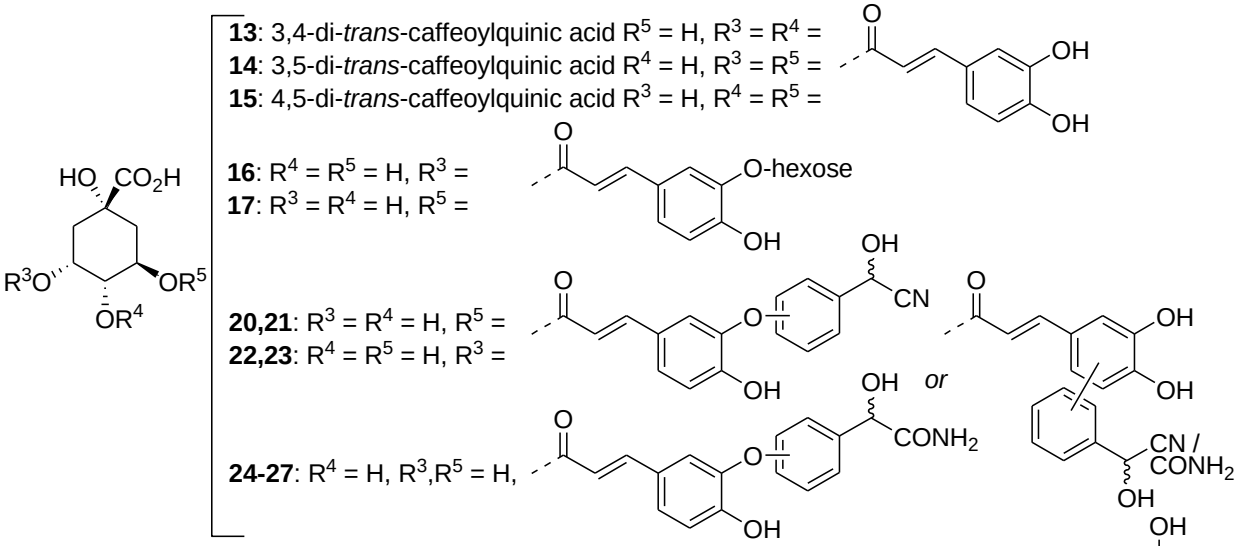
3a: R^1 = OH, 4-*trans*-caffeoylquinic acid
7a: R^1 = H, 4-*trans*-coumaroylquinic acid
11a: R^1 = OMe 4-*trans*-feruloylquinic acid

3b: R^1 = OH, 4-*cis*-caffeoylquinic acid
7b: R^1 = H, 4-*cis*-coumaroylquinic acid
11b: R^1 = OMe 4-*cis*-feruloylquinic acid



4a: R^1 = OH, 5-*trans*-caffeoylquinic acid
8a: R^1 = H, 5-*trans*-coumaroylquinic acid
12a: R^1 = OMe 5-*trans*-feruloylquinic acid

4b: R^1 = OH, 5-*cis*-caffeoylquinic acid
8b: R^1 = H, 5-*cis*-coumaroylquinic acid
12b: R^1 = OMe 5-*cis*-feruloylquinic acid

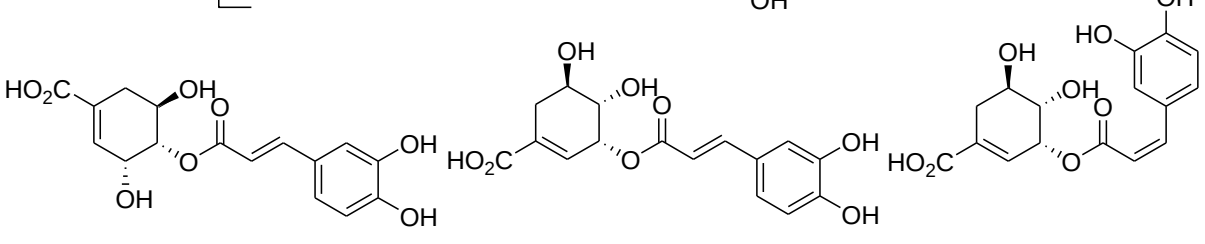


13: 3,4-di-*trans*-caffeoylquinic acid R^5 = H, R^3 = R^4 =
14: 3,5-di-*trans*-caffeoylquinic acid R^4 = H, R^3 = R^5 =
15: 4,5-di-*trans*-caffeoylquinic acid R^3 = H, R^4 = R^5 =

16: R^4 = R^5 = H, R^3 =
17: R^3 = R^4 = H, R^5 =

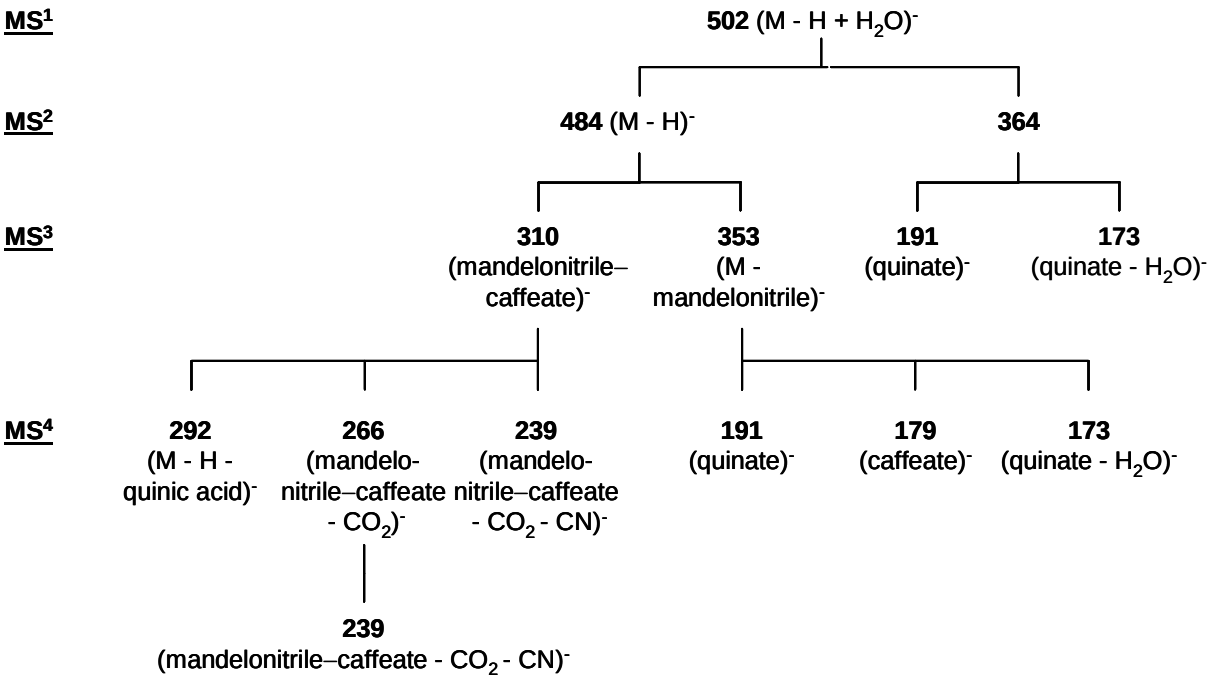
20,21: R^3 = R^4 = H, R^5 =
22,23: R^4 = R^5 = H, R^3 =

24-27: R^4 = H, R^3,R^5 = H,



18: 4-*trans*-caffeoylshikimic acid **19a**: 3-*trans*-caffeoylshikimic acid **19b**: 3-*cis*-caffeoylshikimic acid

Figure 3.


MS1
502 (M - H + H2O)-
MS2
484 (M - H)-
364
MS3
310 (mandelonitrile–caffeate)-
353 (M - mandelonitrile)-
191 (quinate)-
173 (quinate - H2O)-
MS4
292 (M - H - quinic acid)-
266 (mandelo-nitrile–caffeate - CO2)-
239 (mandelo-nitrile–caffeate - CO2 - CN)-
191 (quinate)-
179 (caffeate)-
173 (quinate - H2O)-
239 (mandelonitrile–caffeate - CO2 - CN)-

Scheme 1.

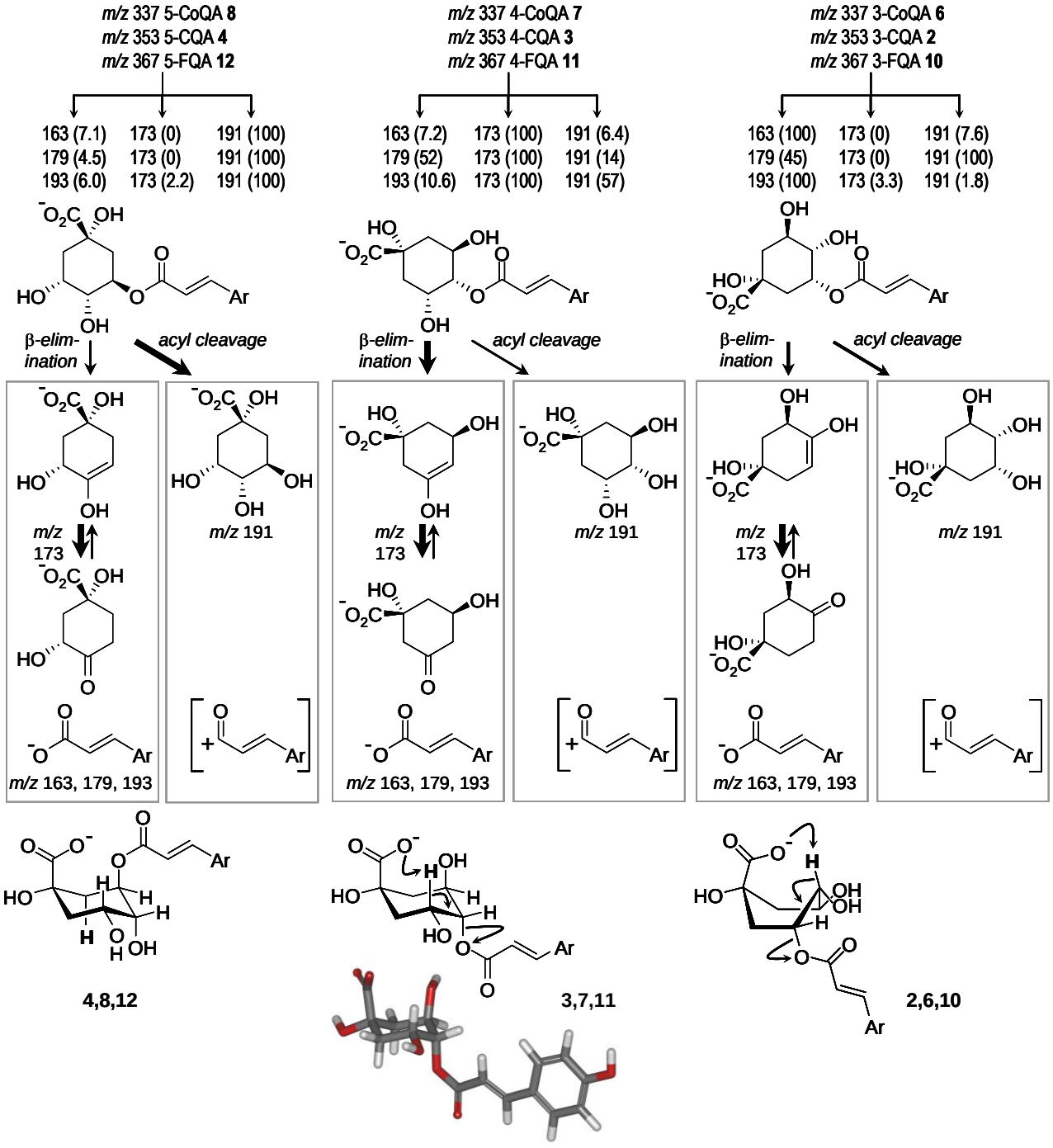

m/z 337 5-CoQA 8
m/z 353 5-CQA 4
m/z 367 5-FQA 12
163 (7.1) 173 (0) 191 (100)
179 (4.5) 173 (0) 191 (100)
193 (6.0) 173 (2.2) 191 (100)
m/z 337 4-CoQA 7
m/z 353 4-CQA 3
m/z 367 4-FQA 11
163 (7.2) 173 (100) 191 (6.4)
179 (52) 173 (100) 191 (14)
193 (10.6) 173 (100) 191 (57)
m/z 337 3-CoQA 6
m/z 353 3-CQA 2
m/z 367 3-FQA 10
163 (100) 173 (0) 191 (7.6)
179 (45) 173 (0) 191 (100)
193 (100) 173 (3.3) 191 (1.8)
β-elim-ination
acyl cleavage
m/z 173
m/z 191
m/z 163, 179, 193
4,8,12
3,7,11
2,6,10

Figure legends

Figure 1. HPLC chromatograms of *Miscanthus* × *giganteus* leak and stem extracts.

Figure 2. Structures of hydroxycinnamates in *Miscanthus* × *giganteus.*

Figure 3. Principal fragmentations of mandelamide-CQA and mandelonitrile-CQA.

Scheme 1. Proposed intramolecular base-driven β-eliminations in the MS^2 fragmentations of O-acyl quinate anions **2-4**,**6-8**,**10-12**. *Upper*: Observed ions in MS^2 negative-ionisation mode; *Middle*: Proposed structures of fragment anions; *Lower*: reacting conformations and MM2-minimised structure for base-driven β-eliminations.

Table 1: Quantification of caffeoyl-, *p*-coumaroyl- and feruloyl- conjugates in the leaves and stems of *Miscanthus* × *giganteus*

Compound (µg/g FW)	**Tissue**	
	Leaf	**Stem**
p-CoQA	17.1	0.6
CQA	349.5	35.8
FQA	12.8	1.0
CQA conjugate (M_r 485)		139.0
CQA conjugate (M_r 503)		7.3

*p*CoQA, *p*-coumaroylquinic acid; CQA, caffeoylquinic acid; FQA, feruloylquinic acid

Table 2: HPLC / UV (340 nm) characterisation of hydroxycinnamates in *Miscanthus* × *giganteus* leaf and stem extracts and negative ion MS^2 fragmentation for caffeoyl- *p*-coumaroyl and feruloyl conjugates.

Peak / Cpd. No.	***m/z* (-ve mode)**	***Compound***	***Geom. isomer***	***MS^2 fragmentation (relative intensity %)***	***HPLC t_R (min)***	***λ_{max} (nm)***	***HPLC t_R (min)***	***λ_{max} (nm)***
					Leaf Extract		**Stem Extract**	
1	353	CQA isomer	?	191 (100), 179 (5.6)	/	/	11.5	323
2a	353	3-CQA	*trans*	191 (100), 179 (45), 135 (6.9)	6.3	323	6.4	n.d.
2b	353	3-CQA	*cis*	191 (100), 179 (44), 135 (7.1)	4.4	n.d.	5.1	n.d.
3a	353	4-CQA	*trans*	191 (14), 179 (52), 173 (100), 135 (5.3)	15.3	326	15.4	323
3b	353	4-CQA	*cis*	191 (31), 179 (55), 173 (100), 135 (5.3)	/	/	13.8	n.d
4a	353	5-CQA	*trans*	191 (100), 179 (4.5), 161 (0.4), 135 (0.6)	12.7	324	12.8	324
4b	353	5-CQA	*cis*	191 (100), 179 (4.6), 161 (0.4), 135 (0.7)	19.5	n.d.	19.5	n.d.
5	337	CoQA isomer	?	191 (100), 163 (8.0)	/	/	17.7	n.d.
6	337	3-*p*CoQA	*trans*	191 (7.6), 163 (100), 119 (3.9)	9.5	n.d.	/	/
7a	337	4-*p*CoQA	*trans*	191 (6.4), 173 (100), 163 (7.2)	20.9	n.d.	20.9	n.d.
7b	337	4-*p*CoQA	*cis*	191 (1.7), 173 (100), 163 (5.7)	15.2	n.d.	/	/
8a	337	5-*p*CoQA	*trans*	191 (100), 163 (7.1)	19.4	n.d.	19.4	n.d.
8b	337	5-*p*CoQA	*cis*	191 (100), 163 (6.6)	26.7	n.d.	26.6	n.d.
9	367	FQA isomer	?	193 (6.4), 191 (100), 173 (5.6)	/	/	22.2	n.d.
10a	367	3-FQA	*trans*	193 (100), 191 (1.8), 173 (3.3), 134 (4.5)	13.2	n.d.	13.3	n.d.
10b	367	3-FQA	*cis*	193 (100), 191 (2.3), 173 (3.7), 134 (4.2)	12.6	n.d.	12.6	n.d.
11a	367	4-FQA	*trans*	193 (11), 191 (57), 173 (100)	25.3	323	25.3	n.d.
11b	367	4-FQA	*cis*	193 (11), 191 (11), 173 (100)	20.2	n.d.	20.1	n.d.

12a	367	5-FQA	*trans*	193 (6.0), 191 (100), 173 (2.2)	24.0	323	24.0	n.d.
12b	367	5-FQA	*cis*	193 (6.1), 191 (100), 173 (2.3)	31.3	n.d.	31.2	n.d.
13	515	3,4-diCQA	*trans*	353 (100), 335 (9.3), 299 (2.5), 203 (2.5), 179 (5.7), 173 (6.9)	42.3	n.d.	/	/
14	515	3,5-diCQA	*trans*	353 (100), 203 (4.8), 191(10)	43.9	n.d.	/	/
15	515	4,5-diCQA	*trans*	353 (100), 317 (6.2), 299 (13), 255 (5.2), 203 (11), 179 (3.2), 173 (4.8)	51.8	n.d.	/	/
16	515	hexosyl-5-CQA	*trans*	455 (100), 479 (45), 424 (38), 395 (28)	9.6	n.d.	/	/
17	515	hexosyl-3-CQA	*trans*	323 (100), 353 (47), 191 (19), 179 (8.3), 173 (4.4)	13.2	n.d.	/	/
18	335	4-caffeoyl-shikimic acid	*trans*	291 (31), 179 (100), 161 (40), 135 (16)	21.2	n.d.	/	/
19a	335	3-caffeoyl-shikimic acid	*trans*	291 (1.8), 179 (100), 161 (1.5), 135 (14)	30.1	n.d.	/	/
19b	335	3-caffeoyl-shikimic acid	*cis*	291 (22), 179 (100), 161 (1.6), 135 (19)	23.8	325	/	/
20	484	mandelo-nitrile-5-CQA	*trans*	353 (14), 310 (68), 292 (95), 266 (16), 265 (100), 191 (26)	/	/	25.7	301, 330
21	484	mandelo-nitrile-5-CQA	*trans*	353 (14), 310 (70), 292 (94), 266 (16), 265 (100), 191 (25)	/	/	27.5	301, 330
22	484	mandelo-nitrile-3-CQA	*trans*	353 (1.9), 310 (100), 292 (17), 266 (12), 265 (7.9), 239 (4.3), 191 (2.1), 173 (3.7)	/	/	30.1	n.d.
23	484	mandelo-nitrile-3-CQA	*trans*	353 (1.4), 310 (100), 292 (15), 266 (12), 265 (6.5), 239 (4.2), 191 (1.8), 173 (3.9)	/	/	32.2	n.d.
24	502	mandelamide-CQA	*trans*	484 (73), 364 (100), 353 (14), 191 (13)	/	/	15.5	302, 332

25	502	mandelamide -CQA	*trans*	484 (100), 364 (67), 353 (16), 191 (7.8)	/	/	17.3	n.d.
26	502	mandelamide -CQA	*trans*	484 (100), 364 (62), 353 (14), 191 (6.9)	/	/	18.9	302, 332
27	502	mandelamide -CQA	*trans*	484 (100), 364 (78), 353 (24), 191 (11)	/	/	19.4	n.d.

n.d. = not detected or masked by co-eluting peaks; *p*CoQA, *p*-coumaroylquinic acid; CQA, caffeoylquinic acid; FQA, feruloylquinic acid; / = not detected in the tissue extract (leaf / stem)